Chapter 5

General discussion

Bacteria and fungi live together in several terrestrial habitats where they have a range of interactions. Knowledge of these interactions is important for a better understanding of terrestrial ecosystem functioning e.g. nutrient cycling, plant nutrition and disease suppression. Nevertheless, the mechanisms and the genetic determinants that underlie bacterial-fungal interactions are still poorly understood. In this PhD study I aimed to improve the understanding of the events and the genes involved in bacterial mycophagy, a trophic interaction in which bacteria feed on living fungi. To achieve this goal I adopted a genomic approach for the study of *Collimonas* bacteria, the first bacteria that were demonstrated to possess mycophagous ability. In this study I addressed the following issues (1) which bacterial genes are up and downregulated when the bacterium is confronted with a fungus and, as a counterpart, which genes are differentially expressed in the fungus as a response to the presence of the bacterium (2) which role does plasmid pTer331 play in the interaction of *C. fungivorans* Ter331 with fungi and in the other phenotypes characterizing *Collimonas* bacteria (3) what is the level of conservation of the genes encoded in the model strain *C. fungivorans* Ter331, especially the genes involved in bacterial-fungal interactions.

Dual expression profiling of the interaction between the bacterium *C. fungivorans* Ter331 and the fungus *Aspergillus niger*

*C. fungivorans* Ter331 shows an antagonistic interaction towards the fungus *A. niger*. When the two organisms are confronted *in vitro* the fungal growth is inhibited and accumulation of bacterial biomass, in the form of slime, can be observed on the plate.
The relationship between antifungal activity and mycophagy has not been clarified, yet, even though it seems that there are common denominators among the two phenomena.

In order to understand the mechanisms and the genetic determinants involved in the antifungal activity of *C. fungivorans* Ter331 against *A. niger* and to elucidate its relationship with bacterial mycophagy, the expression profile of the two organisms during the confrontation was studied *in vitro*. The study resulted in a list of fungal and bacterial genes differentially expressed as a consequence of the confrontation. The fungus stimulated the expression of several bacterial genes, including genes involved in motility, synthesis of exopolysaccharides and of a putative antimicrobial agent, providing evidence for a role played by these activities in bacterial-fungal interactions. The activation of these mechanisms supports also the existence of an overlap between the determinants of antifungal activity and mycophagy. In addition the presence of the fungus activated genes involved in the consumption of fungal derived substrates, suggesting that production of bacterial slime observed on plate may originate from a conversion of fungal biomass into bacterial biomass. We hypothesize that the presence of a fungus coupled with a scarcity of nutrients stimulated the expression of the determinants of mycophagy in *C. fungivorans* Ter331. The fungus responded to the presence of the bacterium by activating genes involved in metabolism of lipid and cell wall. This finding corresponds well with the observation of hyphal deformations such as swelling and hyperbranching. In addition to the medium acidification, which was present also on the control plate, the presence of the bacterium stimulated the expression of genes involved in secondary metabolites, suggesting a possible self-defense reaction of the fungus to the presence of the bacterium. The analysis of differentially expressed genes during this confrontation indicated that both organisms presented signs of distress: the fungus showed upregulation of genes involved in sporulation and endoplasmic reticulum stress and the bacterium showed downregulation of genes encoding ribosomal proteins and upregulation of mobile genetic elements, furthermore both organisms showed sign of nitrogen limitation. Overall, our results indicate that the
interaction between *Collimonas* and *Aspergillus* is characterized by a complex interplay between trophism, antibiosis, and competition for nutrients. The choice of *A. niger* as fungal partner for this study was determined by the fact that this fungus shows a marked inhibition in the presence of *Collimonas*, coupled with a visible accumulation of bacterial biomass. In addition, *A. niger* is well known thanks to its economical and medical relevance and tools such as the genomic sequence and an expression microarray are available for this fungus. This experiment seems to indicate that the fungal reaction to the presence of the bacterium blocks the expression of the full mycophagous potential of *Collimonas*. This might explain the failure to detect an upregulation of the chitinolytic genes, which are expected to be activated when chitin originating from the fungal cell wall is available to the bacterium. Future experiments confronting *Collimonas* with other fungal species will expand our understanding of the genetic determinants of mycophagy.

**Sequence, evolution and function of plasmid pTer331**

Plasmid pTer331 was isolated from its natural host *C. fungivorans* Ter331. Sequencing of the plasmid revealed 91% identity with the sequence of plasmid pIPO2 (196). I compared the sequences of the two plasmids and found that nucleotide substitution and insertion/deletions events were the mechanisms of sequence divergence since pTer331 and PIPO2 split from their common ancestor. Sequence annotation of pTer331 yielded 44 putative genes, mostly involved in replication, partitioning and transfer of the plasmid itself, suggesting that pTer331 is a cryptic plasmid that does not confer any evident phenotypic trait to its host. The failure to detect pTer331 in strains other than *C. fungivorans* Ter331 indicated that the plasmid does not play a role in traits that are common to all *Collimonas* strains, including antifungal activity, mycophagy, weathering and chitinolysis. Afterwards I tested experimentally the hypothesis that pTer331 could confer a selective advantage for the colonization of the plant rhizosphere. This hypothesis was assessed by obtaining a plasmid-free strain and comparing the performance of this strain and the wild type in colonizing the rhizosphere of tomato.
plants. I found that the plasmid had no significant contribution in the rhizosphere competence of \textit{C. fungivorans} Ter331. Thus pTer331 is likely to be a selfish genetic element, maintained in the bacterial host thanks to its ability to self replicate and spread, rather than to the positive effect on the host fitness. Nevertheless the presence on the plasmid of a hot-spot for insertion of additional genetic modules, suggest that this plasmid might incidentally acquire genes useful for the host survival and enhance its survival and spread in the bacterial population. Recently pTer331 has been proposed to be a member of a new family of broad host range plasmids named “PromA” (95). Besides pIPO2 and pTer331, the family includes plasmid pMOL 98 (199), pSB102 (197) and pMRAD02 (287). These five plasmids were isolated from either rhizosphere or soil in distinct locations in The Netherlands, Germany and Japan. The five plasmids show extensive conservation of the plasmid backbone constituted by the genes necessary for plasmid self replication, maintenance and transfer. Van der Auwera and colleagues compared the accessory genes of these five plasmids and found that natural transposons and transposable elements engineered into the plasmids are inserted in the \textit{parA} locus, confirming the presence on plasmid pTer331 of a hot-spot for the insertion of transposable elements (95). The existence of pTer33-related plasmids carrying accessory genes beneficial to their host, supports the hypothesis that this selfish element might constitute a minimized form of PromA plasmids, which, in certain instances, may acquire genes useful for its host and favour their dissemination in the bacterial population. During the confrontation of \textit{C. fungivorans} Ter331 and the fungus \textit{A. niger} (Chapter 3), when the bacterium manifested signs of distress, the upregulation of the plasmid genes was observed. This finding also hints at the possibility that the plasmid plays a role in facilitating the acquisition of new genes useful for bacterial survival in an unfavorable environment.

**Comparative genomic study of \textit{Collimonas} strains**

All \textit{Collimonas} bacteria share characteristics such as the ability to lyse chitin and the ability to feed on hyphae of living fungi, but they differ with
respect to the possession of several traits such as colony morphology and antifungal activity. I investigated the variability in the genomic content of five strains, representatives of the three species formally recognized in the genus *Collimonas*: *C. fungivorans*, *C. pratensis* and *C. arenae*. With the aid of microarray technology I compared the genomic content of the reference strain *C. fungivorans* Ter331 to the genomic content of four tested strains. The genes encoded in the reference genome were divided into two categories: the genes conserved in all strains and the genes conserved in some but not all strains. I expected to find among the genes conserved in all strains the ones determining characteristics common to all *Collimonas* strains and to find among the variable genes the ones responsible for the traits differentiating the *Collimonas* strains from one another. This hypothesis was partially true, indeed genes such as the ones constituting the chitinolytic system were conserved in all strains, in agreement with the fact that chitinolysis is a property characterizing all collimonads. Nevertheless I found that several genes underlying putative determinants of bacterial mycophagy, such as motility, ability to grow on trehalose and secretion systems, were not conserved in all strains. More detailed studies are needed to give evidence that these traits are indeed important for mycophagous growth, e.g. by comparing mycophagous growth yields of different strains or of mutants defective in one of these traits. Given the fact that all *Collimonas* strains are mycophagous, the variability observed in the possession of putative mycophagous determinants is in line with the hypothesis that bacterial mycophagy is a complex phenotypic trait that is built on the possession of several determinants with additive effects, none of which is strictly necessary for the mycophagous phenotype. This hypothesis is also supported by the fact that attempts to trace individual mycophagous genes and gene functions via a loss of function approach were not successful (32). Possession of a variable set of mycophagous determinants may, in addition, explain the variability observed in the interactions between *Collimonas* strains and fungi (30, 34), possibly indicating that different fungal species have variable susceptibility towards various mycophagous determinants.
**Perspectives for future study of Collimonas bacteria**

This thesis has addressed important issues regarding the genetic determinants and the mechanisms involved in the interaction between bacteria and fungi. The study of the confrontation between *C. fungivorans* Ter331 and the fungus *A. niger* yielded a set of genes differentially expressed during this bacterial-fungal interaction. This study confirmed the activation of bacterial mechanisms such as motility and chemotaxis, production of antifungals and degradation of fungal derived substrates and indicated that the fungus reacted with mechanisms of cell self defense and secondary metabolites production. The different sensitivity of the fungus to the determinants activated by the bacterium and the different sensitivity of the bacterium to the strategies of fungal response might determine the outcome of bacterial-fungal interactions and might contribute to the species-specific interaction observed between *Collimonas* strains and fungi, possibly reflecting a niche differentiation among *Collimonas* strains. A comparative genomic study of *Collimonas* strains detected that several potential mycophagous determinants are not present in all strains, suggesting that additional, as yet undetected determinants might be present in different *Collimonas* strains. Exploiting the existence of several mycophagous strains, it is possible to further explore the range of existing mycophagous determinants, as well as the relationship between the set of mycophagous determinants possessed by a bacterium and its interaction with specific fungi. Elucidating the relationship between mycophagous determinants and the susceptibility of target pathogens will open the way for potential application of *Collimonas* strains as biocontrol agents of plant pathogenic fungi.

The availability of non-mycophagous bacterial genera closely related to *Collimonas*, such as *Herbaspirillum* and *Janthinobacterium*, can be exploited for comparative genomic studies aimed at understanding the evolutionary adaptations that led to the mycophagous behavior. The existence of bacterial selected fungal communities and fungal selected bacterial communities supports the existence of a reciprocal influence of bacterial and fungal communities. Future studies on the interactions between...
**Collimonas** and fungi and potential niche differentiation of different **Collimonas** strains will help clarifying the role played by fungi on the evolution of mycophagous traits and the mechanism of bacterial and fungal coevolution. The evolution of mycophagy as a survival strategy should be studied in the context of the relationship of collimonads with other soil microorganisms. Growth of soil microbes is mostly carbon-limited. This will favour the evolution of strategies to exploit new carbon resources (in this case fungal carbon). Hence, collimonad mycophagy may have evolved and be activated to obtain carbon in a carbon-limited environment. Experiments investigating the competitive ability of **Collimonas** for non-fungal substrates will clarify the role played by competition from other soil bacteria in the evolution and activation of **Collimonas** mycophagy.

Currently, it is not clear which environmental conditions determine the outcome of the bacterial-fungal interaction. Future studies to assess the effect of different abiotic conditions applied in the *in vitro* studies will aid in the identification of the conditions that stimulate the activation of the mycophagous behavior. Mycophagy might represent one of the possible trophic strategies of **Collimonas** bacteria. Whether or not **Collimonas** bacteria obtain nutrients from a fungus might depend on the availability of other more accessible food sources and on the proximity of a suitable fungal match. More easily accessible food sources might be preferred when they are available and mycophagy might represent an additional resource in nutrient poor environment.